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Investigating BCL-2 family protein interactions in yeast

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Abstract

Among the different models used to study the biochemical properties and function of proteins of the Bcl-2 family, their heterologous expression in the yeast *Saccharomyces cerevisiae* may look out of place. However, when grown under adequate conditions, yeast cells have mitochondria that have similar properties as those of mammalian cells, and are able to be targeted by mammalian Bcl-2 family members. Yeast thus provides a neutral cellular background to study how proteins of the Bcl-2 family interact with mitochondria, alone or in couple (or more). Most studies done in our laboratory has been done on the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-xL, but yeast can bring useful information about every protein of the family, in terms of their capacity to interact and to regulate the permeabilization of the outer mitochondrial membrane.

1.Introduction

Structure/functions studies of Bcl-2 family members, like for any other proteins, can be done in whole cells or in recombinant models. For the later, one difficulty is to obtain proteins under a conformation as close to the native conformation as possible. This may be a mimitation for a protein such as the pro-apoptotic protein Bax: indeed, Bax exhibits characteristics resembling those of a membrane protein, namely because of the presence of its C-terminal hydrophobic α -helix. For long, investigators have produced the protein deprived from this helix (*e.g.* [1]) or have used detergents to maintain the protein in solution and unaggregated (*e.g.* [2]). However, those surfactants may have the same membrane-permeabilizing effects as active Bax and/or can favor the active conformation of Bax, that maybe a limitation to study its regulation [3].

The heterologous expression of Bcl-2 family members in yeast has been used very early as a middle-term alternative between the expression in mammalian cells and recombinant models. The first direct demonstration that Bax was able to permeabilize mitochondria to cytochrome c has been done in yeast [4], one year before the same demonstration in mammalian cells [5]. Yeast does not have homologs of the Bcl-2 family members, even though a form of programmed cell death is present in yeast ([6] for a review). However, when expressed in yeast, Bcl-2 family members keep their ability to interact with mitochondria and, under adequate conditions, to permeabilize yeast outer mitochondrial membrane to cytochrome c, providing a model to investigate Bax activity ([7] for review).

In this chapter, we describe methods that have been improved for 20 years, that allows to investigate the function of Bax when it is expressed in yeast. These methods have been applied to the study of different Bax mutants [2, 8-12], but can also been applied to the study of combination with other Bcl-2 family members, such as Bcl-xL [12,13], tBid [14], Puma [15], or zebrafish protein Bcl-wav [16]. It can also be used to study the role of other Bax regulation candidates such as Tom22 [17,18] or AKT [11].

2.Materials

Prepare all reagents and buffer in ultrapure water (18 M Ω .cm at 25°C). Buffers containing mannitol and sorbitol should be stored at 4°C. Culture media are autoclaved at 110°C for 20 min.

2.1. Expression system

2.1.1.Plasmids

Because Bcl-2 family members are regulators of life and death, it is necessary to use regulatable expression systems: indeed, the expression of these proteins under the control of constitutively active promoters, may lead to the selection of yeast mutants carrying unwanted alterations (for example, a greater capacity to induce the degradation of these proteins). To prevent this type of bias, cDNA encoding Bcl-2 family members have been cloned under the control of regulatable promoters.

The most popular regulatable promoter in yeast is the GAL1/10 promoter, that is controlled by the addition of the fermentable sugar galactose. It is present on commercially available plasmids such as the pYES series (ThermoFisher) and the pESC series (Agilent). All these plasmids allow to introduce different tags but this is not recommended for Bcl-2 family members, particularly at the C-terminal end, that is crucial to their function.

The GAL1/10 promoter is activated by galactose and inhibited by glucose. Furthermore, glucose is a strong genetic repressor of the expression of mitochondrial proteins, while galactose has a far more moderate effect, namely on respiratory chain components [19, for review]. Shifting yeast cells from glucose to galactose to induce the expression of a protein controlled by GAL1/10 cause both the expression of the protein of interest and the derepression of mitochondrial proteins. This adds a degree of complexity to the observations, thus possibly being a cause of misinterpretations. The correct practice is to grow yeast cells first on glucose (that we call “preculture”), then on a non-fermentable carbon source (lactate, glycerol, ethanol) to derepress mitochondrial proteins, and finally add a moderate concentration of galactose (0.5-0.8%) to induce the expression of the protein of interest. Although less easy to implement, the *tet*-off system might also be useful, in combination with the GAL1/10 promoter, when the sequential expression of two proteins is studied. Also, contrary to the GAL1/10 promoter, the *tet*-off system is not sensitive to the carbon source present in the culture medium and can therefore be used on glucose-grown cells (which is useful to study yeast mutants unable to grow on non-fermentable carbon sources) [20]. Plasmids of the pCM series, which allow to express proteins under the control of the *tet*-off promoter, are available from Euroscarf. The drawback of the *tet*-off system is that the repression is not total. It is therefore better to use it to express anti-apoptotic proteins, that should not impair cell growth.

2.1.2.cDNA

Importantly, the yeast codon bias is different from the mammalian one. A striking example is Glycine, which is encoded by the 4 GGN codons. While the GGG codon is evenly used by mammalian cells (25% frequency), it is almost never used by yeast cells (1% frequency). On the opposite, the GGU codon is the least used by mammalian cells (16% frequency) while it is the most used by yeast cells (47% frequency). Replacing GGG codons by GGU codons thus allowed to increase by 5-10 fold the level of expression of human Bax in yeast cells. It is not required to optimize the gene as extensively as we have done for human Bax (Table 2), but optimizing codons that are very rarely used by yeast may be useful. Free tools are now made available by synthetic genes providers to design such optimization.

2.1.3.Strains

The 3rd partner is the yeast strain itself. The plasmids described above bear at least one of the following auxotrophic marker: *HIS3*, *LEU2*, *TRP1* or *URA3*. Most laboratory yeast strains contain mutations in one or more of these genes, allowing plasmid selection by growing the cells in the absence of the corresponding amino acid or base (histidine, leucine, tryptophan, uracile). Two strains are namely used.

The BY strain series (BY4741, BY4742, BY4743 for the *matA* haploid, *matALPHA* haploid and diploid strains, respectively) is the strain in which the Yeast ORF deletion collection, available from Euroscarf, has been built. However, this strain has a poor mitochondrial metabolism, with about two time less

mitochondrial proteins than the following strain, making it poorly desirable for mitochondria-centered studies.

The W303 strain series (W303-1A, W303-1B, W303 for the *matA* haploid, *matALPHA* haploid and diploid strains, respectively) has been widely used by yeast mitochondria investigators around the world since the 1970's. It exhibits a vigorous growth on non-fermentable carbon sources, connected to a high mitochondria content (at least 25% of the total protein mass when grown on lactate as a carbon source), and bears all 4 auxotrophic markers listed above. The only drawback is that, opposite to the BY strain, there is no centralized collection of mutants, and each laboratory should make its own mutants (or ask to colleagues). However, making a yeast mutant is a relatively easy task, due to the genuinely high ratio of homologous recombination of yeast DNA. We will not detail here the construction of the strains. There are many highest-quality manuals of yeast molecular biology and genetics available, and additional informations can be found in the references given in the introduction.

2.2.Culture Media

1.YNBglu (100 mL). Dissolve 0.17g Yeast Nitrogen Base (Difco), 0.1g potassium dihydrogenphosphate, 0.5g ammonium sulfate, 2g glucose, 10mg of each auxotrophic requirement, in 100 mL water. Adjust the pH at 5.5 with HCl 2N.

2.YNBlac (1L). Dissolve 1.7g Yeast Nitrogen Base, 1g potassium dihydrogenphosphate, 5g ammonium sulfate, 2 g "Drop Mix" (a mixture made with equal weights of 16 aminoacids, except Histidine, Leucine, Lysine, Tryptophan, which are used as selection markers for strains BY and W303), 100 mg of auxotrophic markers, in 200mL water. Add 20g of DL-lactic acid, and adjust the volume to ~500mL. Add 6.5g of NaOH in pellets. When dissolved, finely adjust the pH to 5.5 with HCl 2N or NaOH 2N. Complete the volume at 1L.

Media can be directly put in Erlenmeyer Flasks plugged with cotton for cultures, or stored in Pyrex storage bottles. Sterilize media at 110°C for 20 min. (**see note 1**).

3.Galactose (100mL). Weight 40 g of galactose and add water up to 100mL. Sterilize as above in a Pyrex storage bottle. As soon as the sterilization is done, shake the bottle to dissolve any remaining galactose to prevent further crystallisation. If crystals appear, unscrew the cap and heat in a microwave oven for 2 minutes.

2.3.Buffers for mitochondria preparations

1.Reducing buffer (250 mL) (**see note 2**). Pipette 7.3mL of β -mercaptoethanol in 25mL of Tris 1M. Adjust at 250mL with water. Measure the pH, that should be 9.1-9.3 (adjust with NaOH or HCl, if necessary).

2.KCl/Tris buffer (2L). Weight 75g of KCl. Add 20 mL of Tris 1M. Dissolve in ~1L water. Adjust pH to ~7.0 with HCl. Adjust the volume at 2L.

3.Digestion Buffer (250mL). Prepare 1L of a stock solution of sorbitol 2M. Mix 170 mL of this solution with 10mL of a 0.25M solution of citric acid, and 20mL of a 0.2M solution of Na_2HPO_4 and 2.5mL of a solution of 0.1M EGTA. Adjust the pH to 6.5 with NaOH. Adjust the volume at 250mL with water.

4. Spheroplasts washing buffer (500mL). Prepare 2L of a stock solution of mannitol 0.8M and 500mL of a stock solution of maleic acid 0.5M adjusted at pH 6.8 with Tris. Mix 250mL of the mannitol stock solution with, 187mL of the sorbitol stock solution and 5 mL of Tris/maleate solution. Add 0.5g of BSA. Verify the pH, that should be 6.8 (adjust with NaOH or HCl if necessary).

5. Homogenization and Recuperation buffers (500mL each). Mix 750mL of the mannitol stock solution, 20mL of the Tris/Maleate solution and 20mL of EGTA 0.1M. Verify the pH, that should be 6.8 (adjust with NaOH or HCl if necessary). Adjust the volume at 1L. Keep 500mL that will be Recuperation Buffer. Dissolve 1g of BSA in the other half, that will be Homogenization buffer.

2.4. Other solutions

2.4.1. Cell proteins extraction

Protein extraction. Prepare a stock solution of NaOH 2M. For each set of experiment, mix 35µL of β-mercaptoethanol in 965µL of NaOH 2M.

Protein precipitation. Prepare a stock solution of TCA 3M (**see note 3**).

Sample solubilization. Prepare a solution of 2% SDS in 0.1M NaOH.

3X Laemmli buffer (10mL). Weight 0.6g SDS. Add 1.5 mL of Tris 1M, 0.6mL β-mercaptoethanol, 15mg of Bromophenol Blue. Complete at 7mL with water. Gently mix until all SDS is dissolved. Add 3mL of glycerol. Mix again.

2.4.2. SDS-PAGE

All solutions should be stored at 4°C. Prepare the gel solutions in advance so that the polymerization is done at room temperature. We routinely use the Mini-Protean system from Biorad.

Running (for 2gels): 2.7 mL Acrylamide/bis 37.5/1 40%; 2mL SDS 0.4%, Tris/HCl 1.5M pH 8.8, 3.3 mL water. Add 8µL TEMED and 12µL 40% APS just before casting.

Stacking (for 2 gels): 0.4 mL Acrylamide/bis 37.5/1 40%; 0.8mL SDS 0.4%, Tris/HCl 0.5M pH 6.7, 1.8 mL water. Add 4µL TEMED and 7µL 40% APS just before casting.

Pour the Running gel. Check that there is no leak. Gently add a thin layer of water on top. Wait 30 minutes for polymerization. Remove water with Whatman paper. Pour the stacking gel. Put the combs. Wait at least 2 hours before using the gel (overnight is better). Gels can be stored at 4°C. Pack each gel in wet absorbing paper, and wrap several gels together in plastic food film. If correctly packed, gels can be stored for up to 1 month.

2.4.3. Transfer and Western-Blots

Transfer gels on Nitrocellulose membranes using a standard procedure (we use the Mini-Trans Blot system from Biorad).

Saturate membranes in PBST + dry milk 5%, for 30 minutes at room temperature.

Remove milk.

Put the membrane in an appropriate volume of PBST. Add the primary antibody (see table 3). Incubate overnight at 4°C.

Remove the primary antibody solution (**see note 4**).

Rapidly wash the membrane twice with PBST.

Put the membrane in an appropriate volume of PBST. Add the secondary antibody (see table 3).

Incubate 45 min at room temperature.

Remove the secondary antibody solution (**see note 5**).
Wash the membrane at least six times with 10 min incubation.
Reveal the peroxidase activity with a standard ECL method.

3.Methods

3.1.Mitochondria preparation

- 1.Pregrow yeast cells by suspending a clone in 3mL YNBglu medium in a 20mL glass tube, at 28°C under moderate shaking (180rpm). An overnight culture should be sufficient.
- 2.Transfer 2mL of this preculture in 20mL of YNBlac medium in a 100mL Erlenmeyer flask, at 28°C under moderate shaking (180rpm). The remaining 1mL can be kept and used for re-inoculating a preculture in YNBglu. Follow the growth of the culture by measuring the O.D. at 550nm. The doubling time of the culture is between 4 to 6 hours. The maximal O.D. should not exceed 6 units.
- 3.Transfer the 20mL culture in 180mL of YNBlac medium in a 1L flask, and grow as above (**see note 4**).
- 4.Dispatch the 200mL culture in two 5L-Erlenmeyer flasks containing 1L of YNBlac medium. Grow the cell as above until the O.D. of the culture is 0.7 to 0.9. Add 1:80 volume of the 40% galactose solution (so that the final concentration of galactose in the culture is 0.5%). Grow the cultures under the same conditions as above (**see note 6**).
- 5.Bax-expression controlled by the *GAL1* promoter reaches a plateau after 8 hours. However, to exacerbate the effects on mitochondria and also for practical reasons, it is useful to prolong the expression up to 14 hours (*i.e.* overnight).
- 6.Measure the O.D. of the cultures. 1L of culture at 1 O.D. unit corresponds to about 0.5g dry weight of cells. Save 0.5mL of the culture and kept frozen at -20°C for preparing total extracts. Harvest the rest of the cultures in 1L-centrifugation flasks (4000 x g, 3 min). Resuspend the cells in distilled water, reunite the two pellets, complete at 1L and centrifuge again (4000 x g, 3 min). Resuspend the cells in distilled water, complete at 200 mL and centrifuge in a 250mL-flask (4000 x g, 3 min).
- 7.Resuspend the pellet in 10mL pre-warmed Reducing Buffer per g dry weight. Incubate at 28°C for 15 minutes under gentle agitation (50-100 rpm). (**see note 2**).
During the incubation, weight the adequate amount of zymolyase (10mg per g dry weight) and dissolve it in the adequate volume of pre-warmed digestion buffer (10mL per g dry weight).
- 8.Centrifuge the cells (4000 x g, 2 min). Resuspend the cells in the KCl solution (**see note 7**).
Centrifuge (4000 x g, 2 min), resuspend in KCl solution, and centrifuge again (4000 x g, 2 min).
- 9.Resuspend the pellet in the zymolyase-containing Digestion buffer prepared at step 7. When cells are resuspended, dilute 20µL of the suspension in 1mL water and measure the O.D. at 550nm: this is the reference value for intact cells. Incubate the cells at 28°C under gentle agitation (50-100rpm). Every 10 minutes, measure the O.D. of the suspension, as above. Because of the digestion of their wall, digested cells swell and splinter when diluted in water, and the O.D. of the suspension decreases. When the O.D. reaches 20% of the reference value, cells are considered as completely digested. (**see note 8**). If the experiment has been correctly done, the digestion should be complete after 30-40 minutes. In any case,

do not prolong the digestion beyond 50 minutes because cells that are already digested - and their content - might be altered.

Important: all subsequent steps should be done on ice or, even better, in a cold room.

10. Transfer the suspension of digested cells in 30-mL centrifuge tubes (SS34-type). Do not fill the tubes at more than half. Centrifuge (5000 x g, 5 minutes). Gently resuspend the pellet in the Spheroplast Washing Buffer, using the teflon-made pestle of a Potter. The pellet of digested cells should be soft and easy to resuspend. If the pellet is sticky, this means that cells were not correctly digested. Centrifuge, resuspend, centrifuge, resuspend and centrifuge, all in the same conditions as above.

11. Resuspend the cells in an adequate volume of homogenization buffer. Starting from a 2L culture at 2 O.D. unit (2 g dry weight), 20-25 mL is an adequate volume. Adjust the volume to the initial amount of cells. Pour the suspension in a Waring blender stainless steel minicontainer (MC1 or MC2, i.e. 37ml or 110mL capacity), and homogenize with 3 pulses of 3 seconds each.

12. Dilute the suspension at least 4-fold with the homogenization buffer, and dispatch in 30mL-centrifugation tubes.

13. Centrifuge at low speed (800 x g, 15 min). Transfer carefully the supernatant in fresh tubes, and centrifuge at high speed (17,000 x g, 15 min).

14. Resuspend each mitochondrial pellet in a small volume of the recuperation buffer (1-2mL). Gently homogenize 3 times in a 5mL Potter-Elvehjem homogenizer equipped with a soft Teflon pestle. Dilute the mitochondrial suspension to 20-25mL with recuperation buffer.

15. Repeat step 13.

16. Resuspend the mitochondrial pellet in the smallest possible volume of recuperation buffer (0.5 - 1.5mL). Gently homogenize in the Potter-Elvehjem homogenizer.

17. Optional: Yeast mitochondria can be frozen without significant alteration of their permeability properties, provided precautions are taken. Place a flat recipient in a flat polystyrene box. The recipient should be as smooth as possible, without asperity. The cut bottom (2-3 cm height) of a standard 2kg plastic container of a chemical product is adequate. Pour liquid nitrogen in both the recipient and the polystyrene box (**see note 9**).

18. Pour the mitochondria suspension in a 5mL-syringe equipped with a 26 Gauge needle. Push very gently on the piston so that small droplets of the suspension fall in liquid nitrogen. Move the syringe so that droplets do not “fuse” to each other. Keep your needle far enough from the liquid nitrogen surface so that the suspension does not freeze inside.

19. When all the suspension is frozen as small beads, collect and store them in a cryotube (pre-cooled in liquid nitrogen). Store the cryotubes in a -80°C freezer (**see note 10**). Frozen mitochondria can be stored for up to 1 year.

20. **Thwaving.** Prepare a Becher containing hot water. It should be hot, but not boiling: 65-70°C. Put the adequate amount of mitochondria beads in a standard 5mL hemolysis glass-tube (**see note 11**). Put

the tube in hot water for several seconds with gentle agitation. As soon as the suspension is thawed, put the tube on ice. (see note 12).

21. Make a protein dosage on the mitochondria suspension. If necessary, dilute the sample to work in the range of the protein dosage).

3.2.Mitochondrial/Cellular Bax ratio

The purpose of this experiment is to quantify the amount of Bax in mitochondria compared to the total amount of Bax.

1. Use the sample of cell culture, that has been saved in Point 6 of paragraph 3.1. Based of the O.D. value, calculate the cell concentration (1mL of culture at 1.O.D. unit contains 2×10^7 cells). Centrifuge a volume equal to 10^7 cells (10,000 x g, 1 min). Resuspend the pellet in 0.5 mL water. Add 50 μ L of a mixture of β -mercaptoethanol 3.5% (v/v) in NaOH 2M. Vortex. Incubate on ice for 30 min. Add 50 μ L of TCA 3M. Vortex. Incubate on ice for 15 min. Centrifuge (10,000 x g, 2 min). Carefully remove the supernatant with a pipette. Add 200 μ L of acetone. Centrifuge (10,000 x g, 2 min). Carefully remove the supernatant with a pipette. Let the pellet dry at room temperature. Add 30 μ L of a solution of 2% SDS in 0.1M NaOH. Solubilize the pellet (use bath sonication if necessary). Add 15 μ L of Laemmli buffer 3X. Incubate the tubes at 70°C for 15 min. Samples are ready for SDS-PAGE.

2. Use a volume of mitochondria suspension (as obtained in 3.1) corresponding to 0.5mg protein. Complete to 100 μ L with water. Add 10 μ L of TCA 3M. Vortex. Incubate on ice for 15 min. Centrifuge (10,000 x g, 2 min). Carefully remove the supernatant with a pipette. Add 75 μ L of acetone. Centrifuge (10,000 x g, 2 min). Carefully remove the supernatant with a pipette. Let the pellet dry at room temperature. Add 20 μ L of a solution of 2% SDS in 0.1M NaOH. Solubilize the pellet (use bath sonication, if necessary). Add 10 μ L of Laemmli buffer 3X. Incubate the tubes at 70°C for 15 min. Samples are ready for SDS-PAGE.

3. Load cellular and mitochondrial extracts from the same culture on the same SDS-PAGE gel, so that it will be possible to quantify the mitochondrial/cellular ratio. Transfer gels on nitrocellulose membranes, for western-blotting. Use an antibody against Bax and an antibody against a yeast mitochondrial protein, such as porin. After revelation and quantification of the bands, calculate the following ratio for each experimental condition:

$$\frac{(\text{Bax in mitochondria}/\text{Porin in mitochondria})}{(\text{Bax in cells}/\text{Porin in cells})}$$

3.3.Bax insertion in MOM

1. For each condition, prepare 4 microfuge tubes containing 0.5mL of Recuperation Buffer. Add the following solution to each tube:

Tube 1: 50 μ L of Recuperation Buffer (control)

Tube 2: 50 μ L of Na₂CO₃ 1M, pH 10 (extraction of peripheral proteins)

Tube 3: 50 μ L of Triton X-100 1% (extraction of membrane-anchored proteins)

Tube 4: 50 μ L of Triton X-100 10% (extraction of membrane-inserted proteins)

2. Adjust the mitochondrial suspension at 10mg/mL with recuperation Buffer.

3. Add 50 μ L of the mitochondrial suspension (0.5mg) to each tube. Mix gently. Incubate on ice for 15 min. Centrifuge (22,000 x g, 20 min).

4. Carefully transfer the supernatants in fresh tubes. Add 50 μ L of TCA 3M and proceed for the preparation of samples for electrophoresis like described above (3.2, point 1).

5. Resuspend the pellets in 100 μ L water. Add 10 μ L of TCA 3M and proceed for the preparation of samples for electrophoresis like described above (3.2, point 2).

6. Load supernatants and pellets samples on different SDS-PAGE gels (**see note 13**). After transfer on nitrocellulose, do western-blots against Bax and different mitochondrial proteins (Porin, Cox2, cytochrome c,...). After quantification, compare the resistance of Bax v/s the different treatments, to that of other proteins.

3.4. Cytochromes quantification

The spectrophotometric quantification of cytochromes can be done on whole cells or isolated mitochondria. Bax activation leads to the permeabilization of MOM and the release of cytochrome c in the cytosol. Consequently, isolated mitochondria only contain unreleased cytochrome c. By comparing to mitochondria isolated from control cells, it is possible to measure the ability of Bax to permeabilize MOM.

The release of cytochrome c is associated to an instability of the protein, that is more rapidly degraded than the mitochondrial protein. Consequently, yeast cells where cytochrome c is released contain less cytochrome c than control cells. Although this is less precise than measurements on isolated mitochondria, it is less material-consuming and far more rapid, and can be used for a rapid screening of different conditions, before a more precise measurement on isolated mitochondria

We will describe both methods.

3.4.1. Cytochromes quantification on isolated mitochondria

1. Prepare 2mL of a mitochondria suspension at 5mg/mL in Recuperation buffer (**see note 14**).

2. Dispatch the suspension in 1mL spectrophotometric cuvettes (**see note 15**). Load the cuvettes in the Cary 4000 spectrophotometer (**see note 16**). Make an acquisition between 650 and 500 nm to check that spectrophotometer settings are adequate: the signal should be more or less horizontal and close to 0.

3. Add several mg of potassium ferricyanide (oxidant) in the Reference cuvette and several mg of sodium hydrosulfite (reducer) to the Sample cuvette (**see note 17**). Mix well by inverting the cuvettes several times.

4. Make an acquisition between 650 and 500nm. The spectrum should have the general shape reported in Figure 1, with three peaks around 603 nm, 561 nm and 550 nm, and three wells at 630nm, 575nm and 540nm.

5. Note the O.D. values at those 6 wavelengths. Calculate the differences at 603 nm *minus* 630nm (α), 561 nm *minus* 575 nm (β), and 550 nm *minus* 540nm (γ), corresponding to cytochrome a+a3, b and c+c1, respectively. Also calculate the value 575 nm *minus* 540 nm (δ), to do a graphical correction of spectrum misshapes.

6. Calculate the concentration of the three cytochromes (in mM), including the graphical corrections:
Cytochrome a+a3: $\alpha/24$

Cytochrome b: $(\beta + 2\delta/5)/18$

Cytochrome c+c1: $(\gamma - 2\delta/7)/18$

7. Convert molar concentrations of cytochromes to pmoles/mg mitochondrial proteins.

8. Since cytochrome b is an unreleased membrane-inserted protein, it is an internal control to the experiments. The ratio cytochrome c+c1/cytochrome b is therefore a measurement of Bax ability to release cytochrome c. The maximal ratio may vary from experiment to experiment, depending on mitochondria preparation, but is typically 2.0 +/- 0.2. The minimal ratio (if cytochrome c is fully released and only cytochrome c1 remains) is 0.5 (that is the stoichiometry between cytochrome c1 and cytochrome b within Respiratory Complex III).

Typically, we consider that a ratio above 1.7 corresponds to the absence of release, a value between 1.2 and 1.5 corresponds to a moderate release, and a value below 1.0 corresponds to a strong release.

3.4.2. Cytochromes quantification on whole cells

1. Make a cell culture (paragraph 3.1, points 1-5) in a volume large enough to get 2mL of cells at 100 O.D. units (i.e. 100 mL of culture at 1 O.D. unit, or 50 mL of culture at 2 O.D. units, or 200 mL of culture at 0.5 O.D. units, etc..).

2. Centrifuge the cells (5,000 x g, 5 min). Resuspend the pellet in the appropriate amount of water to obtain 50 O.D. units.

3. Dispatch the suspension in the two cuvettes of the spectrophotometer. Make an acquisition between 650 and 500 nm to check that spectrophotometer settings are adequate: the signal should be more or less horizontal and close to 0, but is expected to be more noisy than the measurements on isolated mitochondrial.

4. Add 2µL of Hydrogen Peroxide (30 % vol) (oxidant) in the Reference cuvette and several mg of sodium hydrosulfite (reducer) to the Sample cuvette (*see note 18*). Mix well by inverting the cuvettes several times.

5. Do the acquisition and calculations like on mitochondria suspension (paragraph 3.4.1., points 4-8).

Notes

1. It is crucial that the temperature of sterilization does not exceed 110°C) (note: sterilized YNB media can be stored for 1-2 weeks without special precautions. However, since some components are light-sensitive, longer periods of storage should be done away from light.

2. The reducing buffer contains a high concentration of β-mercaptoethanol, that has an unpleasant smell and is toxic. If possible, work under a chemical hood and, in any case, work in a ventilated area.

3. As a very hygroscopic solid acid, pure TCA may cause very severe burns. Always use gloves, lab coat, eyes protection, and weight it with extreme caution. It is less hazardous once in solution, but it should nevertheless be manipulated with caution.

4. It may be worthy to keep primary antibodies solutions for re-use. Antibodies should be added in PBST or TBST without BSA nor milk. For short storages at 4°C (one week), it is not necessary to add a

preservative. For long storage, most antibodies solutions can be frozen and thawed at least once without significant activity loss. Considering their low cost, it is not worth to reuse secondary antibodies.

5.: it is crucial to use transient cultures in Erlenmeyer flasks of intermediate size, and to maintain a 1 to 5 ratio between the volume of culture and the volume of the flask. As a matter of fact, do not inoculate directly a 1L culture with a 20mL preculture, because the dilution effect will induce a long lag time before yeast cells start to grow again.

6.two liters of culture is the minimal comfortable volume to obtain mitochondria of good quality. It is possible to isolate mitochondria from 1L-cultures but their quality will be poorer.

7.At this stage, the cells should become “sticky” and be more difficult to resuspend. Use a spatula.

8. Alternatively, digestion can be followed under a light microscope at x400 magnification. On a glass plate, mix 2 μ L of cell suspension and 10 μ L of water. Digested cells appears swollen, dark-colored, and possibly splintered. I personally prefer this method. It requires some experience, but is often more precise than the O.D., that may give erroneous measures, particularly when cells are stuck together.

9.Standard safety procedures should apply when manipulating liquid nitrogen, to avoid frostbites.

10.Do not close completely the caps of the cryotubes, until all liquid nitrogen has evaporated.

11.It is crucial to use glass tubes, for better heat conductance.

12.This thawing step is crucial, and requires some experience. Proceed with small amounts of beads, so that the whole preparation is not lost in case of mis-manipulation.

13.The reason for this is that, due to the presence of residual mannitol, supernatant samples often result in “dirty” blots.

14.This is the optimal concentration. However, a reasonable precision can be obtained with suspensions at a concentration above 3 mg/mL.

15.It is better to use black walled cuvettes to limit diffusion

16.The crucial factor is to have a spectrophotometer able to depict a small signal from a high basal level due to light diffusion. This is why a true dual monochromator equipped spectrophotometer, such as the Varian Cary 4000, is required.

17.The precise amount may depend of the degree of purity, but 5mg is a good starting point. Do not prepare solutions of these products, because they tend to be rapidly degraded in solution.

18.Potassium ferricyanide cannot be used on whole cells because it is not permeant.

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| Plasmid | Promoter | Selection marker | Provider |
|----------------|--|------------------------------|-----------------|
| pYES2/CT | <i>GAL1</i> | <i>URA3</i> | ThermoFisher |
| pYES3/CT | <i>GAL1</i> | <i>TRP1</i> | ThermoFisher |
| pYES6/CT | <i>GAL1</i> | bsd (blasticidin resistance) | ThermoFisher |
| pESC-HIS | <i>GAL1/10*</i> | <i>HIS3</i> | Agilent |
| pESC-LEU | <i>GAL1/10*</i> | <i>LEU2</i> | Agilent |
| pESC-TRP | <i>GAL1/10*</i> | <i>TRP1</i> | Agilent |
| pESC-URA | <i>GAL1/10*</i> | <i>URA3</i> | Agilent |
| pCM184 | <i>tet-off (tetO₇/CYC1)</i> | <i>TRP1</i> | Euroscarf |
| pCM189 | <i>tet-off (tetO₇/CYC1)</i> | <i>URA3</i> | Euroscarf |

Table 1: Plasmids that have successfully been used to express Bcl-2 family members in yeast.

*Both *GAL1* and *GAL10* promoters are present in opposite directions, with two distinct multicloning sites.

Native

ATG GAC GGG TCC GGG GAG CAG CCC AGA GGC GGG GGG CCC ACC AGC
TCT GAG CAG ATC ATG AAG ACA GGG GCC CTT TTG CTT CAG GGT TTC
ATC CAG GAT CGA GCA GGG CGA ATG GGG GGG GAG GCA CCC GAG CTG
GCC CTG GAC CCG GTG CCT CAG GAT GCG TCC ACC AAG AAG CTG AGC
GAG TGT CTC AAG CGC ATC GGG GAC GAA CTG GAC AGT AAC ATG GAG
CTG CAG AGG ATG ATT GCC GCC GTG GAC ACA GAC TCC CCC CGA GAG
GTC TTT TTC CGA GTG GCA GCT GAC ATG TTT TCT GAC GGC AAC TTC
AAC TGG GGC CGG GTT GTC GCC CTT TTC TAC TTT GCC AGC AAA CTG
GTG CTC AAG GCC CTG TGC ACC AAG GTG CCG GAA CTG ATC AGA ACC
ATC ATG GGC TGG ACA TTG GAC TTC CTC CGG GAG CGG CTG TTG GGC
TGG ATC CAA GAC CAG GGT GGT TGG GAC GGC CTC CTC TCC TAC TTT
GGG ACG CCC ACG TGG CAG ACC GTG ACC ATC TTT GTG GCG GGA GTG
CTC ACC GCC TCA CTC ACC ATC TGG AAG AAG ATG GGC TGA

Optimized for yeast expression

ATG GAC **GGT** TCC **GGT** **GAA** **CAA** **CCA** AGA **GGT** GGG **GGT** **CCA** ACC **TCC**
TCT **GAA** **CAA** ATC ATG AAG **ACT** **GGT** GCC **TTG** TTG CTT **CAA** GGT TTC
ATC **CAA** **GAC** **AGA** GCT **GGT** **AGA** ATG **GGT** **GGT** **GAA** **GCT** **CCA** **GAA** **TTG**
GCC **TTG** GAC **CCA** **GTT** **CCA** **CAA** GAC **GCT** TCC ACC AAG AAG **TTG** **TCT**
GAA TGT **TTG** AAG **AGA** ATC **GGT** GAC GAA **TTG** GAC **TCT** AAC ATG **GAA**
TTG **CAA** **AGA** ATG ATT GCC **GCT** **GTC** GAC **ACC** GAC TCC **CCA** **AGA** **GAA**
GTC **TTC** TTC **AGA** **GTC** GCC GCT GAC ATG **TTC** TCT GAC **GGT** AAC TTC
AAC TGG **GGT** **AGA** **GTT** GTC GCC **TTG** TTC TAC **TTC** GCC **TCT** AAG **TTG**
GTC **TTG** AAG GCC **TTG** TGT ACC AAG GTC **CCA** GAA **TTG** ATC AGA ACC
ATC ATG **GGT** TGG **ACT** TTG GAC TTC **TTG** **AGA** **GAA** **AGA** **TTG** TTG **GGT**
TGG ATC CAA GAC **CAA** GGT GGT TGG GAC **GGT** **TTG** **TTG** TCC TAC **TTC**
GGT **ACT** **CCA** **ACT** TGG **CAA** ACC **GTT** ACC ATC **TTC** **GTT** GCC **GGT** **GTC**
TTG ACC GCC **TCT** **TTG** ACC ATC TGG AAG AAG ATG **GGT** TGA

Table 2: cDNA sequences of native human Bax (top) and substitutions (in bold) made to obtain the yeast-optimized cDNA (bottom).

| Antigen | Antibody/Company | Host | Dilution |
|-------------------------------|--|-------------|-----------------|
| Human Bax | N20 / Santa-Cruz | Rabbit | 1/5,000 |
| Human Bax | 2D2 /Santa-Cruz | Mouse | 1/2,000 |
| Human Bcl-xL | E18 /Abcam | Rabbit | 1/10,000 |
| Yeast Porin | 16G9E6BC4 /Thermofisher | Mouse | 1/50,000 |
| Yeast Phosphoglycerate kinase | 22C5D8 /Thermofisher | Mouse | 1/10,000 |
| Yeast Cytochrome c | Custom antibody raised against purified yeast cytochrome c from Sigma-Aldrich (C-2436) | Rabbit | 1/5,000 |
| Mouse IgG | Peroxidase-Coupled/ Jackson | Goat | 1/10,000 |
| Rabbit IgG | Peroxidase-Coupled/ Jackson | Goat | 1/10,000 |

Table 3: List of antibodies that have been routinely used for studying Bax and Bcl-xL expression in yeast, and adequate controls.